ECOLOGY AND GENOMICS OF *DICKEYA SOLANI*, A NEW SOFT ROT BACTERIUM INFECTING POTATOES

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ACADEMIC DISSERTATION

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ISNN 2342-5423 (Print)
ISNN 2342-5431 (Online)
ISBN 978-951-51-1513-3 (Paperback)
ISBN 978-951-51-1512-6 (PDF)
http://ethesis.helsinki.fi
Hansaprint 2015

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ABSTRACT

This dissertation is aimed at studying the emergence and invasiveness of a new soft rot bacterium: *Dickeya solani*. Members of the *Dickeya* genus cause soft rot and blackleg in potato, which is one of the world's most important food crops. *D. solani* was first isolated in Finland in 2004; since then, it has been identified in many European countries. It most likely emerged in the Netherlands and spread elsewhere in Europe and beyond via the potato seed trade, and it has rapidly became a major threat for potato production, along with the other commonly known soft rot bacteria *D. dianthicola* and *Pectobacterium* species. In this study, the ecological aspects and genomics of *D. solani* were investigated to elucidate the mechanisms underlying its fast spread and high aggressiveness.

A real-time PCR test was developed to analyze plant samples containing *Dickeya* species. This test ensures the fast determination of the amount of *Dickeya* cells present in symptomatic plant tissue and avoids lengthy and labored procedures for bacterial cell isolation, thereby representing an advantageous diagnostic tool.

Biovar 3 *Dickeya* isolates were biochemically characterized and found to differ from the other known *Dickeya* species isolated from potatoes and ornamentals in Europe. Taxonomic analysis indicated that these strains belonged to a new species within the genus *Dickeya,* which was consequently named *D. solani*.

The genome of a Finnish *D. solani* strain was sequenced to study the invasive character of this new species. Comparative genomic analysis indicated the presence of a set of specific genes involved in the production of toxic secondary metabolites, which triggered the investigation of the ability of *D. solani* to outcompete other bacterial species present in the potato environment. A newly identified bacteriocin-like molecule produced by *D. solani* promoted the growth inhibition of some *Pectobacterium* species, thus partly explaining the rapid colonization and dominance of the potato environment by this novel soft rot pathogen.

This study provides novel information about the ecology, taxonomic status and genomics of the new potato pathogen *D. solani*. This information is likely to contribute to applied studies to improve potato production and plant health in general in the future.

LIST OF PUBLICATIONS

This dissertation is based on original publications and some previously unpublished data that are reprinted and can be found at the end of this book.

The following original publications are reproduced with the permission of the copyright holders:

I. **Laurila J, Hannukkala A, Nykyri, J, Pasanen M, Hélias V, Garlant, L, Pirhonen M.** 2010. Symptoms and yield reduction caused by *Dickeya* spp. strains isolated from potato and river water in Finland. Eur J Plant Pathol **126**:249-262.

II. **van der Wolf JM, Nijhuis EH, Kowalewska MJ, Saddler GS, Parkinson N, Elphinstone JG, Pritchard L, Toth IK, Lojkowska E, Potrykus M, Waleron M, de Vos P, Cleenwerck I, Pirhonen M, Garlant L, Helias V, Pothier JF, Pfluger V, Duffy B, Tsror L, Manulis S.** 2013. *Dickeya solani* sp. nov., a pectinolytic plant pathogenic bacterium isolated from potato (*Solanum tuberosum*). Int J Syst Evol Microbiol **64**(3):768-74.

III. **Garlant L, Koskinen P, Rouhiainen L, Laine P, Paulin L, Auvinen P, Holm L, Pirhonen M.** 2013. Genome Sequence of *Dickeya solani*, a New soft Rot Pathogen of Potato, Suggests its Emergence May Be Related to a Novel Combination of Non-Ribosomal Peptide/Polyketide Synthetase Clusters. Diversity **5:**824-842.

IV. **Garlant L, Liu Y, Nykyri J, Hannukkala A, Pirhonen M.** 2015. A toxic molecule produced by the potato soft rot bacterium *Dickeya solani* inhibits the growth of pectobacteria. Manuscript submitted.

The publications are cited in the following text with the correspondent roman numbers.

ABBREVIATIONS

ABC	ATP-binding cassette
AHL	*N*-Acyl homoserine lactone
ANI	Average nucleotide identity
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CDI	Contact dependent inhibition
Ct	Cycle threshold
CVP	Cristal violet pectate
DDH	DNA-DNA hybridization
DNA	Deoxyribonucleic acid
DSMO	Dimethyl sulfoxide
FAS	Fatty acid synthase
Hcp	Hemolysin-coregulated protein
HGT	Horizontal gene transfer
IGS	Intergenic spacer
MDR	Multidrug resistance
MIM	Minimal inducing medium
MLSA	Multilocus sequence analysis
MS	Mass spectroscopy
NCBI	National Center for Biotechnology Information
NRPS	Non-ribosomal protein synthase
ORF	Open reading frame
PCR	Polymerase chain reaction
PCWDE	Plant cell wall-degrading enzyme
Peh	Polygalacturonases
Pel	Pme pectate lyases
PEM	Pectate enrichment medium
PFGE	Pulsed field gel electrophoresis
PI	Pathogenicity island
PKS	Polyketide synthase
Pme	Pectin methylesterases
Pnl	Pectin lyases
rDNA	Ribosomal DNA
REP-PCR	Repetitive Sequence-Based PCR
Rhs	Rearrangement hotspot

R-M	Restriction-modification
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system
T6SS	Type VI secretion system
TPS	Two-partner secretion
WGS	Whole genome sequence

Keywords: plant pathology, bacteriology, soft-rot enterobacteria, *Dickeya*, *Pectobacterium*, potato soft-rot and blackleg, genomics, bacterial interactions.

1. INTRODUCTION

Plant pathology is a science that investigates the nature, cause and control of plant diseases. New pathogens can be found on diseased plants that show new symptoms with no previous records or that harbor a disease agent that cannot be identified by reference to special manuals. Plant bacteriology studies the disease-causing agents represented by plant pathogenic bacteria.

Bacterial isolation, detection and identification are fundamental steps for the study of the ecology and pathogenesis of plant pathogenic bacteria. Isolation of bacteria from the infected tissue can be achieved using specific culture methods, but isolation is not always possible. Molecular plant pathology developments have provided a new set of diagnostic tools and techniques based on biochemical and DNA analysis that enable the detection of pathogens even when they are present in very small numbers or mixed with other closely related pathogens. The identification of plant pathogenic bacteria relies on taxonomic classifications that are continuously evolving as new tools are developed to investigate relationships and diversity among bacteria.

At present, whole genome sequencing has become an efficient and cost-effective method for the identification and characterization of plant pathogens. Genomics provides a solid platform for the development of standards for taxonomy and detailed knowledge of gene functions and structures (Konstantinidis *et al.*, 2006; Chun and Rainey, 2014). This important data asset can be used to shed light on the molecular mechanisms characterizing a bacterium´s pathogenesis and virulence and its interactions with its host and other organisms (Demuth *et al.*, 2008). The same information is employed in ecological studies to understand the spread, establishment and survival of a bacterial pathogen in a given environment (Shapiro et al., 2009; Wilson, 2012). Finally, the overall investigation of plant pathogenic bacteria relies on gathering information that can be used to develop new disease control strategies to protect crops against bacterial diseases.

This thesis focuses on a new soft rot bacterial pathogen of potatoes: *Dickeya solani*. Studies on diagnostic methods, taxonomic status, genomics and bacterial interactions of *D. solani* are discussed.

1.1. Importance of potato production

The potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world, after maize, wheat and rice. It was most commonly grown and consumed in developed areas such as North America and Europe until 2005, when the developing country China became the biggest potato producer. Currently, Asia is the world´s major potato-producing region (followed by Europe) and leads potato consumption *per capita* (FAO, 2008; IPC, 2015).

Potato plants belong to the genus *Solanum*. They are annual herbaceous plants that produce tubers that are very rich in starch. After the growing season when the aerial parts of the plant die, the tubers allow the plant to survive the cold and reproduce during the following favorable season by mean of buds on the tuber that generate shoots.

Potato tubers are composed of 80% water and 20% dry matter, the latter consists of 60 to 80% starch. The tubers are low in fat and their protein content is similar to cereals based on dry weight. They are rich in vitamin C and are a good source of vitamins B1, B3 and B6, minerals and antioxidants (NNDSR, 2015).

Over two-thirds of potatoes produced in 2005 were consumed by people as food (FAO, 2008). When they are not processed in food products, fresh potatoes are boiled, baked or fried to overcome the inability of human beings to digest the starch in row tubers. Up to 96% of the starch can be extracted from raw potatoes and used for the distillation of alcoholic beverages, or used as a thickener, adhesive, binder, and substitute for polystyrene and other plastics and to produce fuel-grade ethanol. Each year, an important 5 to 15% of the crop is used as “seed potatoes” for the next planting season.

Potatoes are a “cool weather crop” with optimum yields between 18 and 20˚C, but are also grown under temperate, subtropical and tropical conditions. Potato plants are usually grown from seed potatoes, small tubers or pieces of tuber sown 5 to 10 cm deep in the soil. Seed tuber selection based on quality and disease-free certification is essential for a successful crop. The Netherlands is the world's biggest supplier of certified seed potatoes (FAO, 2008).

Potatoes require between 500 to 700 mm of water during the period of time between planting and harvest (which lasts, on average, between 120 and 150 days). The crop reaches maturity when the tubers can be easily separated from their stolons and the leaves turn yellow. The potato tubers are living tissues; thus harvesting procedures must avoid bruising or other injury and storage conditions must provide darkness, temperatures lower than 8˚C and high relative humidity (85 to 90%). These precautionary measures are taken to ensure the well-being of the potato tuber and avoid the development of bacterial diseases that are the major threat to potato production (van der Wolf and De Boer, 2007).

1.2. Blackleg and soft rot diseases of potatoes

Bacteria are always present when fleshy plant tissues rot in the field or in storage. Some of the bacteria are not pathogenic and may be saprophytic or secondary parasites; others are necrotrophic bacteria that attack living tissue and cause soft rot leading to huge losses of fleshy vegetables and ornamentals worldwide (Agrios, 2005).

Pectobacterium and *Dickeya* species are among the best characterized potato pathogens. They cause foliage diseases known as blackleg, aerial stem rot and stem wet rot, as well as soft rot in tubers (van der Wolf and De Boer, 2007). Blackleg disease caused by *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis*, *P. wasabiae* and *Dickeya* species appears as a slimy, wet and black rot lesion that spreads from the rotting mother tuber to the stems under humid conditions. Symptoms under dry conditions include stunting, yellowing, wilting and desiccation of the stems and leaves. Aerial stem rot that develops from the top and progresses downward to the base is caused by the above-mentioned species, including *P. carotovorum* subsp. *carotovorum,* late in summer under persistent wet conditions (Pérombelon and Kelman, 1980) because the bacteria are dispersed with water and aerosols.

When potato tubers are affected by soft rot, their tissue is macerated to a creamy consistency that turns black in the presence of air. The bacteria enter the potato tuber tissue through lenticels, stolons and/or wounds, and the infection can spread to the whole plant. Tubers become more susceptible to soft rot development with high levels of moisture that increase the incidence and spread of the disease and low oxygen levels that promote infection because these pathogens are facultative anaerobes (Pérombelon, 2002).

Potato tuber soft rot can occur in the field or during storage, leading to the non-emergence of the potato plant if the mother tuber is infected, and massive rotting in the stored tuber lot when liquid leaking out from rotting tubers infects the neighboring tubers (Czajkowski, 2011).

Bacterial soft rot and blackleg infections in potatoes generally begin with asymptomatic infected seed tubers (Pérombelon and Lowe, 1975). Some *Pectobacterium* and *Dickeya* species can survive in other infected hosts, spread to potato plants (Dickey, 1980; Ma *et al.*, 2007) and overwinter in infected plant remnants in the soil. Soft rot bacteria can be released into the soil from rotting tubers, infected root systems and infected haulms or plant remnants, especially during rainfall (Pérombelon, 1982). They can move in the soil to a distance up to 10 m by means of free water (Graham and Harper, 1967), thus possibly infecting neighboring plants. Bacteria can also be dispersed by insect vectors from diseased plants to other potato crops and can be present in the aerosols formed by rain impaction on symptomatic plants and by haulm destruction prior to harvest. Furthermore, soft rot pathogens are found in surface water and can be dispersed in potato crops by irrigation practices (Laurila *et al.*, 2008). Potato harvesters may become contaminated by contact with rotting tubers and spread the disease to the next harvest. Moreover, the machinery used during harvesting may provoke wounds in the tubers that can easily be infected by contact with ubiquitous rotting tuber materials during handling and grading procedures (Czajkowski, 2011).

Disease control is difficult and relies mainly on prevention measures such as avoiding plant injuries and contamination during harvesting and handling, assuring good insect control and practicing crop rotation. Maintaining good storage conditions helps the tuber tissue stay dry and cool, thereby

providing unfavorable condition for the development of tuber soft rot (Czajkowski, 2011). The production and utilization of pathogen-free certified seed potatoes reduce the bacterial load on tubers, and thus the chance of the plant developing soft rot disease. Furthermore, biological control agents such as *Serratia plymuthica* A30 have been selected against soft rot and blackleg pathogens (Czajkowski *et al.*, 2012).

Diseases associated with potatoes have a significant economic impact. Economic losses are caused by blackleg in potato plants that leads to the rejection of contaminated seed tubers and soft rot of potato tubers in the field and in post-harvest conditions, such as shipment and storage. The potato soft rot causing agents *Pectobacterium* and *Dickeya* species are listed among the ten most important bacterial pathogens (Mansfield *et al.*, 2012). Under these circumstances, the study of soft rot pathogens is essential to develop better disease control strategies that can be beneficial to farmers, potato industries and final consumers.

1.3. Taxonomy and description of potato soft rot-causing agents

Bacillus carotovorus was the first pathogen to be related to the soft rot of stored vegetables in 1901 by L. R. Jones. Forty-four years later, this pathogen was identified and renamed *Pectobacterium carotovorum* (Kado, 2010). In 1969, the name of this pathogen was changed to *Erwinia carotovora* var. *carotovora* (Dye, 1969), and subsequently new reclassifications re-established *Pectobacterium* as a new genus and created a second new genus (*Dickeya*) that contained soft rot-causing bacterial species (Hauben *et al.*, 1998; Samson *et al*., 2005). The *Dickeya* genus was named after microbiologist Robert S. Dickey and contained bacteria previously included in *Erwinia chrysanthemi* species (Samson *et al.*, 2005).

Dickeya and *Pectobacterium* species (also known as “soft rot *Erwinia*” and “soft rot *Enterobacteriaceae*”) are Gammaproteobacteria belonging to the Enterobacteriaceae family. They are small Gram-negative bacteria characterized as non-spore-forming straight rods that are motile with peritrichous flagella. These soft rot potato pathogens are facultative anaerobic necrothrops that are defined as opportunistic pathogens because they cause latent infections that evolve into disease when potato resistance is impaired. *Pectobacterium* and *Dickeya* species also secrete a variety of cell wall-degrading enzymes that macerate the plant tissues, leading to plant cell lysis and the release of cellular fluids (a typical soft rot symptom) (Hauben *et al.*, 1998; Samson *et al*., 2005).

P. carotovorum subsp. *carotovorum* and *P. atrosepticum* were the first potato tuber soft rot-causing agents to be identified within the *Pectobacterium* genus. Recently, *P. wasabiae* (Pitman *et al.*, 2008) and *P. carotovorum* subsp. *brasiliensis* (Duarte *et al.*, 2004) were also found to infect potato tubers. In addition to tuber soft rot, *P. carotovorum* can cause aerial stem rot in potatoes; this pathogen has a broad host range worldwide (Pérombelon, 2002). *P. atrosepticum*, *P. wasabiae* and *P. carotovorum* subsp. *brasiliensis* are considered to be blackleg pathogens. *P. atrosepticum* infects

potatoes mainly in temperate regions (Pérombelon, 2002); *P. carotovorum* subsp. *brasiliensis* has been reported to cause severe disease in potatoes in tropical and subtropical regions since 2004 (Duarte *et al.*, 2004; van der Merwe *et al.*, 2010; De Boer *et al.*, 2012; Panda *et al.*, 2012; Nunes Leite *et al.*, 2014; Onkendi and Moleleki, 2014); *P. wasabiae*, previously reported to cause disease in the horseradish, was found in potatoes from several countries in 2008 (Pitman *et al.*, 2008; Nykyri *et al.*, 2012). However, there was evidence that *P. wasabiae* infected potato plants in Europe and North America (Nykyri *et al.*, 2012; Pasanen *et al.*, 2013; Waleron *et al.*, 2013; Khayi *et al.*, 2014) prior to 2008, but was misidentified as *P. carotovorum* subsp. *carotovorum*. The presence of *P. carotovorum* subsp. *brasiliensis* in potatoes prior to 2004 has also been misrepresented. The subspecies *brasiliensis* was misidentified as *P. carotovorum* subsp. *carotovorum* due to the lack of sensitivity of the detection methods used, which were based on the species classification in force at that time. Thanks to new, more accurate techniques, *P. carotovorum* subsp. *brasiliensis* isolates were proven to be present in potatoes prior to 2004 in different continents, including North and South America and Asia (Duarte *et al.*, 2004; Kim *et al.*, 2009; Lee *et al.*, 2014). Recent results suggest it is also present in Europe (Nunes Leite *et al.*, 2014).

The genus *Dickeya* was described more recently and it comprises 7 species (*D. aquatica*, *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. paradisiaca*, *D. solani* and *D. zeae*) and two subspecies (*D. dadantii* subsp. *dadantii* and *D. dadantii* subsp. *dieffenbachiae*). Most of these species and subspecies correspond to the biovar and pathovar classifications of *Erwinia chrysanthemi* (Samson *et al.*, 2005). The genus represents several changes in taxonomy (Figure 1) and is now tending towards consolidation thanks to the unambiguous comparative analysis of whole genome sequences of all species and strains.

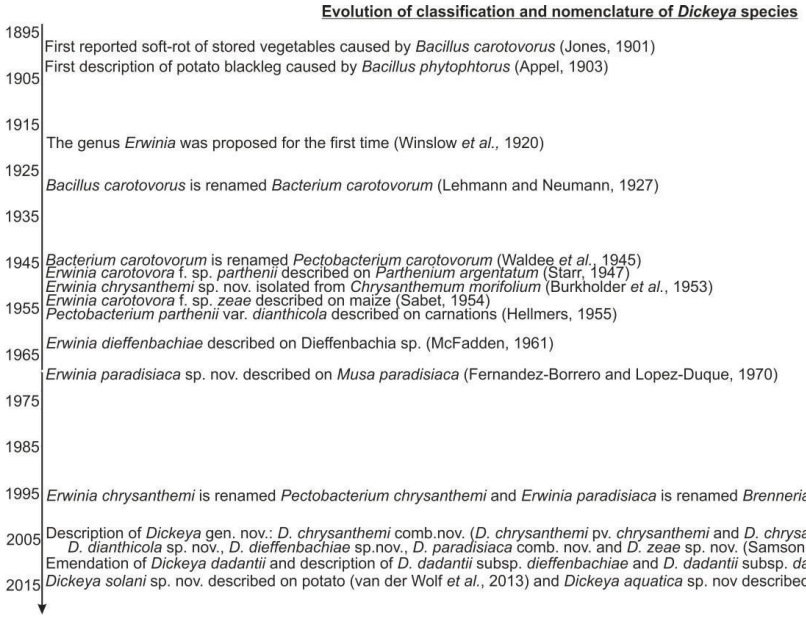

Figure 1. Evolution of the classification and nomenclature of *Dickeya* species. Changes in the taxonomy of the *Dickeya* genus, species and subspecies since the first soft rot pathogen was reported in 1901.

Although the host range of *Dickeya* species is relatively broad and is associated with infections in many different plants, strains from all species and subspecies (with the exception of *D. aquatica*) have been isolated from potatoes. *D. solani* and *D. dianthicola* are the only *Dickeya* species isolated from potatoes in Europe. Additionally, *D. solani* was isolated in Georgia (Tsror *et al.*, 2011) and Israel (Tsror *et al.*, 2009), *D. chrysanthemi* was isolated from potatoes in the USA, Taiwan (Parkinson *et al.*, 2009; Slawiak *et al.*, 2009) and Israel (Lumb *et al.*, 1986), *D. dadantii* was isolated in Brazil (Parkinson *et al.*, 2009), Peru (DeLindo, 1978; Slawiak *et al.*, 2009) and Zimbabwe (Ngadze *et al.*, 2010), and *D. zeae* was isolated in Australia and Papua New Guinea (Cother, 1980; Cother *et al.*, 1992; Parkinson *et al.*, 2009; Slawiak *et al.*, 2009). Pathovars *zeae* and *dianthicola* of *D. chrysanthemi* were also isolated from potatoes in South Africa (Serfontein *et al.*, 1991). However, comparison of *Dickeya* species distributions on potatoes worldwide is often problematic because species identifications can be incorrect. As phylogenetic analyses have evolved into more precise tools for species classification, strain identification has become more reliable. Thus, erroneous strain identifications have been corrected due to the availability of more specific DNA analyses. An example is the reclassification of *Dickeya* strains Ech586, Ech703 and Ech1591 that were used as wrong reference strains in several studies for many years until their misclassification was revealed (Marrero *et al.*, 2013).

1.4. Emergence of *Dickeya solani*

Dozens of plant species in both the monocotyledon and dicotyledon groups of Angiosperms have been described as hosts for *Dickeya* species (Ma *et al.*, 2007). In Europe, all *Dickeya* spp. (except *D. paradisiaca* and *D. aquatica*) were detected in ornamentals, and the first record of *Dickeya* in potato dated back to 1972 (Maas Geesteranus, 1972). The earlier isolates were later classified as *D. dianthicola*, a *Dickeya* species that was considered to have spread presumably to potatoes in Europe from *Dianthus,* for which it is considered as quarantine pathogen (Toth *et al.*, 2011). *Dickeya* spp. were considered to be pathogenic in warm and tropical areas (Pérombelon, 2002). However, *Dickeya* strains have also been identified in cooler regions since 2004. These strains were first isolated in Finland from diseased potato stems (Laurila *et al.*, 2008). From 2005 onwards, they were also detected in the Netherlands (Czajkowski et al., 2009b), UK (Parkinson *et al.*, 2009), France (Helias, 2012), Poland (Sławiak *et al.*, 2009), Georgia (Tsror *et al.*, 2011), Israel (Tsror *et al.*, 2009) and other countries in Europe (Figure 2) (Potato Council, 2014).

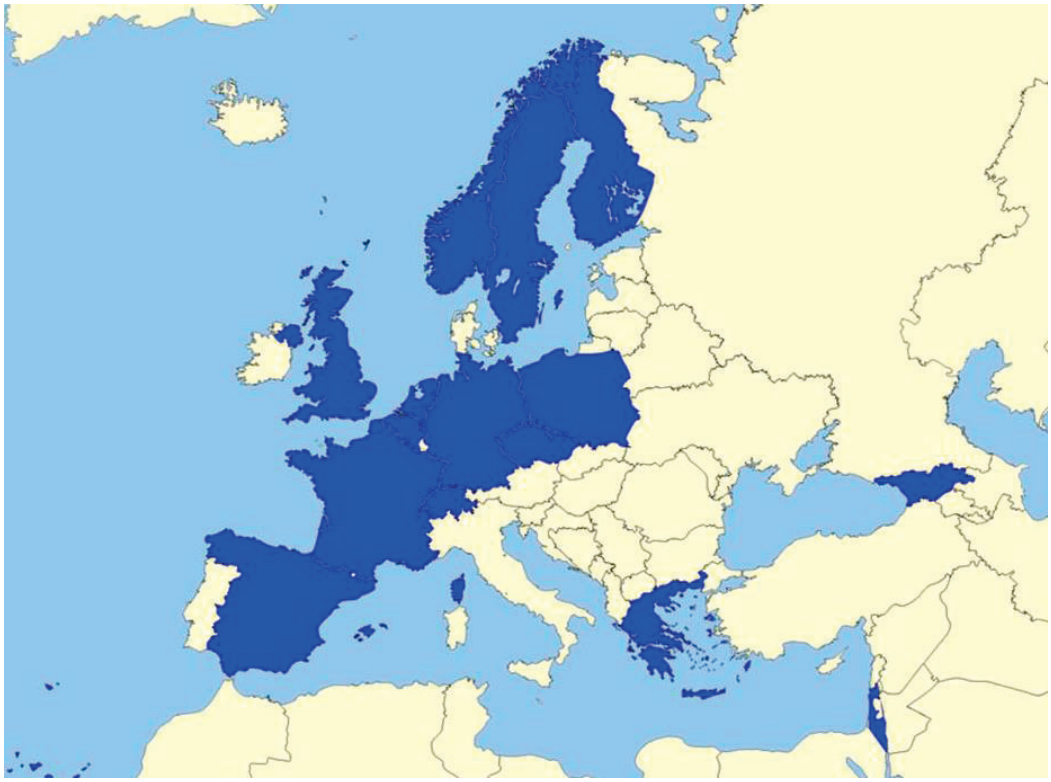

Figure 2. Distribution of *Dickeya solani* on potatoes in Europe. *D. solani* strains were isolated from potatoes in the countries marked in blue.

All of the isolates from the different countries appeared to be clonal and resembled a Dutch strain isolated from hyacinth (Slawiak *et al.*, 2009). Furthermore, a new strain was isolated from plants

grown from Dutch seed potatoes in Israel (Tsror *et al.*, 2009). This finding suggested that this new *Dickeya* strain might have originated as a hyacinth pathogen and spread across Europe via the seed potato and hyacinth tuber trade (Chen *et al.*, 2014). The new strains were identified as biovar 3 according to their biochemical characteristics (Samson *et al.*, 1987; Ngwira and Samson, 1990) but did not cluster with any of the classified species at the time, suggesting the formation of a new species with the proposed name *Dickeya solani* (Toth *et al.*, 2011). Since its emergence in Europe in 2004, this new, highly aggressive *Dickeya* sp. has been responsible for significant potato crop losses.

The virulence of *D. solani* appears to be higher based on the degree of pathogenicity and symptom severity of *D. solani* strains compared with *Pectobacterium* strains that infect potatoes under similar conditions (Toth *et al.*, 2011). *Dickeya* spp. infecting potatoes have higher optimal growth temperatures (Janse and Ruissen, 1988), require a lower amount of inoculum to cause disease symptoms (van der Wolf *et al.*, 2007), and are better colonizers of vascular tissue compared to other *Pectobacterium* species (Czajkowski et al., 2010a). In particular, *D. solani* can infect the roots from a soilborn inoculum, causing blackleg and the systemic colonization of the potato plant (Czajkowski et al., 2010a). Furthermore, *D. solani* is able to move downwards in the vascular tissue of the infected potato plant and colonize the roots, stolons and progeny tubers (Czajkowski et al., 2010b).

While potato tuber soft rot caused by *D. solani* is indistinguishable from the rot caused by other *Dickeya* or *Pectobacterium* spp., the disease symptoms on potato stems can slightly differ. *D. solani* causes potato stem internal soft rot. In this condition, the stem base appears to be healthy, but the symptoms can spread higher up the stems, leaves and petioles, leading to wilting and eventually blackleg symptoms (Toth *et al.*, 2011)(Figure 3).

The aggressive character of *D. solani* has enabled this pathogen to take the place of the other *Pectobacterium* and *Dickeya* species that were previously identified as the primary potato soft rot pathogens.

Figure 3. Disease symptoms on potato stems caused by *D. solani*.

1.5. Potato plant infection and virulence mechanisms of *Dickeya*

Similar to other soft rot bacteria, *Dickeya* spp. enter the potato plant tissue through wounds or lenticels and spend most of their parasitic life in the apoplast (the nutrient-limited space between plant cells). During the first latent infection phase, necrothropic and opportunistic phytopathogens such as *Dickeya* species seem to imitate a biothrophic life style, evading or suppressing the host defenses. When conditions are favorable, the latency period ends and the bacteria begin to multiply. The presence of water on the tubers is considered to be the main factor ending latency (Pérombelon and Lowe, 1975) because it causes the formation of a water film (Burton and Wigginton, 1970) that deprives the tuber tissues of oxygen. Anaerobiosis impairs the tuber resistance system and inhibits cell wall lignification and suberization, which are indispensable defenses against plant cell wall-degrading enzymes (PCWDEs) (Pérombelon, 2002). PCWDEs secreted by soft rot bacteria cause cells to separate from one another, leading to maceration and softening of the infected tissue. Once the plant cells shrivel and their walls are dissolved, they are finally invaded by bacteria and the disease becomes well-established (Pérombelon, 2002).

PCWDEs and toxins represent virulence determinants that characterize the “brute force” lifestyle of soft rot bacteria in the active necrotrophic phase of their life cycle. The production and secretion of most PCWDEs involved in pathogenesis are controlled by small molecules produced by both bacterial cells and plant host cells, such as pectic fragments, plant organic acids and *N*-acyl homoserine lactone (AHL) (Charkowski *et al.*, 2012). The secretion of PCWDEs occurs through several alternative pathways. Proteases that break down plant cell wall proteins and degrade enzymes are secreted via a type I secretion system (T1SS) (Charkowski *et al.*, 2012). *Dickeya* spp. secrete several proteases that may not play a crucial role in pathogenesis, but may provide amino acids for the biosynthesis of microbial proteins (Toth *et al.*, 2003). PCWDEs secreted by type II

secretion systems (T2SS) consist of cellulases, xylanases and pectinases with different properties. At least two cellulases in *Dickeya* spp. (CelZ and CelY) are involved in the plant attack in combination with other exoenzymes, but are not essential for pathogenesis (Boccara *et al.*, 1994). These enzymes hydrolyze 1,4-beta-D-glucosidic bonds in the cellulose in the primary and secondary cell walls of the host plant due to their endobetaglucanase activity (Aymeric *et al.*, 1988). Xylanase secreted by *Dickeya* monocot strains aids the plant tissue maceration process by breaking down 1,4-beta-xylan in the hemicellulose of the plant cell wall (Hurlbert and Preston, 2001). Pectinases specifically target the pectin matrix of the plant cell wall and are the main cause of tissue collapse, cell damage and leakage and disease development in general. Pectinases include pectate lyases (Pel), pectin lyases (Pnl), pectin methylesterases (Pme), and polygalacturonases (Peh); their presence, production, regulation and secretion differ between *Pectobacterium* and *Dickeya* species (Pérombelon, 2002). The production of pectinases, especially Pels, is crucial for pathogenicity. However, in *Dickeya* spp. the same enzymes can be differentially produced in different situations (e.g., in different hosts), thus contributing diversely to virulence (Beaulieu *et al.*, 1993). *Dickeya* spp. generally possess five major Pels, divided into two families (Pel A, D, E and Pel B, C), and at least four secondary Pels (Pel I, L, Z and X) (Hugouvieux-Cotte-Pattat *et al.*, 1996; Pissavin *et al.*, 1996; Shevchik *et al.*, 1997; Shevchik *et al.*, 1999). *Dickeya* pectinases cleave pectin polymers in small fragments which are imported into the bacterial cells and catabolized to 2-keto-3-deoxygluconate and carbon, providing energy sources for cell growth (Hugouvieux-Cotte-Pattat *et al.*, 1996).

Soft rot bacteria have evolved several strategies to colonize the apoplast and overcome host resistance. Among these mechanisms, the type III secretion system (T3SS) and its effectors play a crucial role in the bacteria-plant interaction. The T3SS is essential for virulence and is responsible for the secretion and translocation of harpins into the plant cell. Harpin proteins in *Dickeya* are aggregating factors that contribute to pathogen adhesion in the host plant (Yap *et al.*, 2006). The T3SS system is also involved in the delivery of DspE, the only known effector encoded by *P. carotovorum,* which induces cell death in potato plant leaves (Kim *et al.*, 2011). Homologs of DspE are also found in some *Dickeya dadantii* and *D. solani* genomes (Hogan *et al.*, 2013). The flagellar apparatus that provides motility to the bacterial cell and enables it to react to chemotactic stimuli is considered a subtype T3SS. Moreover, flagellin in *Dickeya* flagella stimulates plant cell defense mechanisms, thereby eliciting plant cell death (Charkowski *et al.*, 2012). The elicitation of the hypersensitive response (HR) in the plant cell can eventually turn into disease because plant cell death is a favorable condition for the initiation of infection by necrotrophic bacteria (Davidsson et al., 2013). *Dickeya* and *Pectobacterium* species can also elicit necrosis on potatoes by secreting most likely through T2SS necrosis-inducing virulence proteins (Nip) belonging to the growing necrosis and ethylene inducing protein-1 (Nep1) family that are also found in other microbial plant pathogens (Mattinen *et al.*, 2004).

Bacterial cells require iron to maintain their biological activity during host plan invasion. Iron in plant tissues is not freely available because it is associated with iron-transporting ligands. Thus, *Dickeya* spp. secrete siderophores (high affinity iron chelating molecules) to sequester iron from the host plant and import it into the bacterial cell. *Dickeya* spp. produce and secrete at least two different siderophores: chrysobactin and achromobactin (Franza *et al.*, 2005).

1.6. Detection of *Dickeya* and *Pectobacterium* species

Symptomatic plant tissue contains high numbers of bacteria (over 10^6 cells per gram of tissue), enabling easy spotting and sampling of the area in which the soft rot bacteria are growing. However, the bacterial density is lower in latently infected tissues and the symptoms are not evident (Czajkowski *et al*., 2014). In latently infected potato plants, bacteria are more commonly found in the first 15-20 cm of stem above the ground (Hélias *et al.*, 2000) and in the stolon ends (Czajkowski et al., 2009a), lenticels and suberized wounds in the tubers (Pérombelon, 2002). When the number of bacteria is low (i.e., in asymptomatic tissues), it might be necessary to enrich the pathogen population prior to proceeding to detection. Liquid pectate enrichment medium (PEM) and anaerobic conditions can be used to enrich the pectinolityc bacteria present in the sample (Pérombelon and van der Wolf, 2002). The isolation of single viable bacterial cells is usually best achieved by plating the sample on semi-selective Crystal Violet Pectate plates, followed by incubation at 27˚C for 48 hours (Pérombelon and van der Wolf, 2002). The crystal violet in this medium inhibits the growth of Gram-positive bacteria; polypectate (which is used as the sole carbon source as in PEM medium) is degraded by pectinolityc bacteria and forms cavities around their colonies, thereby facilitating their isolation (Pérombelon and Burnett, 1991).

The identification of the isolated bacterial colonies can be accomplished through bioassays relying on the different biochemical and serological characteristics of each species and subspecies. Detection of *Dickeya* and *Pectobacterium* species is based on their biochemical profiles; for example, all of the species produce catalases but not oxidases and they all are able to metabolize carbohydrates under both anaerobic and aerobic conditions (Czajkowski *et al.*, 2014). Only species that belong to *Dickeya* are able to produce indole from tryptophan, possess phosphatase activity, are sensitive to erythromycin and are unable to produce acid from trehalose (Czajkowski *et al.*, 2014). These biochemical abilities can be used in biochemical tests (Pérombelon and Hyman, 1986; Pérombelon and van der Wolf, 2002) to discern *Dickeya* from *Pectobacterium* strains. Furthermore, biovars, that sometimes go aside with *Dickeya* species delineation, can be identified by their ability to grow at 39°C, utilize arabinose, melibiose, raffinose, mannitol, tartrate, and inulin, hydrolyze arginine and produce 5-ketogluconate (Samson *et al.*, 1987; Ngwira and Samson, 1990; Palacio-Bielsa *et al.*, 2006). *Pectobacterium* strains can also be identified by testing their growth at 37°C, in 5% NaCl, on lactose, melibiose, raffinose or sorbitol, their production of reducing sugars from

sucrose and their utilization of α-methyl glucoside (De Boer and Kelman, 2001; Pérombelon and van der Wolf, 2002).

Methods that base bacterial detection and identification on the isolation and testing of viable cells from environmental samples are costly and time consuming, especially when high numbers of samples need to be analyzed (i.e., seed potato certification schemes). Molecular techniques based on the detection of nucleic acids overcome these limitations because they do not require living cells, are highly specific and reproducible and provide fast qualitative and quantitative detection of bacteria even from samples with high microbial backgrounds (Rastogi and Sani, 2011). These methods consist of the amplification of a specific target DNA sequence by polymerase chain reaction (PCR). The template DNA can be extracted from previously isolated bacterial colonies, complex environmental samples or from mixed cultures obtained by enrichment. The specificity of the PCR reaction depends on the primer pair used, which can be designed to target sequences at the genus, species or subspecies level. For example, the amplification and sequencing of the 16S rDNA and 16S-23S rDNA intergenic spacer region (IGS) with primer pairs 27f/L1r and1491f/L1r, respectively (Fessehaie *et al.*, 2002), allows the creation of phylogenetic trees with different *Pectobacterium* and *Dickeya* species clustering in distinct groups.

In addition to bacterial identification, quantification of targeted pathogens can also be achieved by real-time PCR. In this DNA amplification procedure, the template is quantified each cycle by measuring the fluorescence of a flurophore in the reaction excited at the required wavelength. Fluorophores can consist of fluorescent dyes that intercalate the double-stranded DNA amplified during each cycle (i.e., SYBR Green) or nucleotidic probes labelled with a fluorescent reporter that is detected after the probe hybridizes the target amplified sequence (i.e., TaqMan). Real-time PCR provides simultaneous identification and quantification of *Pectobacterium* and *Dickeya* species directly from plant material. All *Dickeya* species can be detected by real-time PCR using the TaqMan primers ECHf and ECHr (Pritchard *et al.*, 2013). The TaqMan primer pairs DIA-Af/DIA-Ar and DIA-Cf/DIA-Cr (Pritchard *et al.*, 2013) allow the detection of *D. dianthicola* strains, while *D. solani* strains can be identified using the TaqMan primer pairs SOL-Cf/SOL-Cr, SOL-Df/SOL-Dr (Pritchard *et al.*, 2013) and dsf/dsr (Van Vaerenbergh *et al.*, 2012).

1.7. Bacterial interactions in the potato environment

To become established in the potato environment, a bacterial species needs to compete for nutrients and space with other saprophytic bacteria present in the same niche (Lund, 1979). The competition of several microbial species for the same ecological niche is the driving force for the evolution of weapons enabling one bacterial strain to outcompete and dominate in the potato tuber environment.

Gram-negative bacteria use the TonB-dependent transport system to acquire compounds (i.e., carbohydrates, nickel, vitamin B12, and iron complexes) across the outer membrane. *Dickeya* and

Pectobacterium strains possess TonB-dependent outer membrane receptors and TonB homologs that enable them to use diverse exogenous siderophores that confer enhanced competitiveness for resource uptake, and thus achieve higher fitness in multiple niches (Schauer *et al.*, 2008).

Polyketides and non-ribosomal peptides are among the biological weapons used by bacteria to defeat other microbes. These peptides are secondary metabolites produced by bacterial and fungal polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs), respectively. PKS and NRPS are multidomain enzymes that link acyl-CoAs and amino acids, respectively, through a cascade of condensation reactions (Donadio *et al.*, 2007). Their products are ecologically important due to their various biological activities, such as adaptation to unfavorable environments and competition with other microbes. Several *Dickeya* genomes harbor genes with PKS and NRPS domains (Pedron *et al.*, 2014). A compound called zeamine produced by PKS enzymes of *D. zeae* was shown to be a potent phytotoxin and antibiotic (Zhou *et al.*, 2011).

During competition with secondary invaders, soft rot bacteria must be able to defend themselves from plant, bacterial and fungal toxins. Antimicrobial compounds are transported outside the bacterial cell via multidrug resistance (MDR) systems. *Dickeya* spp. possess different MDR transport proteins that allow them to cope with different toxins, and thus to survive in hostile host environments (Valecillos *et al.*, 2006).

Some pathogenic bacteria are able to target other bacterial species or strains and deliver toxins that inhibit their growth. This system is called contact-dependent growth inhibition (CDI) and is mediated by the CdiB/CdiA two-partner secretion (TPS) system (Ruhe *et al.*, 2013). The transmembrane transporter CdiB translocates the exoprotein CdiA to the inhibitory cell surface where CdiA can bind to the receptor on the target cell. This binding triggers the translocation of CdiA-CT into the target cell, where it causes growth inhibition. This system also includes CdiI, an autoimmune protein that protects the toxin-producer strain from self-inhibition (Aoki *et al.*, 2010). CDI systems are encoded by numerous bacterial species with major differences in CdiA-CT and CdiI sequences. The N-terminal portion of CdiA homologs usually contains hemagglutination activity domains (Pfam domain PF05860), a number of hemagglutinin repeat domains (Pfam PF05594) and a Pfam domain of unknown function (DUF638; Pfam PF04829). *Dickeya* species harbor systems such as Tps and Rhs (rearrangement hotspot) that share action modes and structures with CDI systems; toxins are usually delivered by type V (T5SS) or VI (T6SS) secretion systems (Poole *et al.*, 2011; Pedron *et al.*, 2014). In *D. dadantii* 3937, two Tps systems are composed of the TpsB outer membrane protein (CdiB), TpsA toxin (CdiA-CT) and CdiI immunity protein; these systems are conserved in similar genomic locations in *D. solani* 3337. The *D. dadantii* 3937 and *D. solani* strains also harbor three Rhs systems, including the Rhs protein (CdiA) and RhsI immunity protein (CdiI) that are linked to the hemolysin-coregulated protein (Hcp) and valine-glycine repeat protein G (VgrG) that encode the components of the external part of the T6SS (Pedron *et al.*, 2014). The modules and locations of the

same toxin/antitoxin systems in different *Dickeya* species are usually conserved, except for the homologs of the C-terminal end of CdiA (representing the toxin portion containing the specific growth inhibitory activity) and CdiI, thereby ensuring distinct growth inhibition mechanisms (Aoki *et al.*, 2010; Poole *et al.*, 2011; Pedron *et al.*, 2014).

In addition to CDI, certain Gram-negative enterobacteria produce bacteriocins and microcins that can inhibit other bacteria. These are proteinaceous toxins that can kill closely related bacterial species, thereby enhancing the competitiveness of the producer strain and allowing its establishment in the environment (Rebuffat, 2012). The toxins enter the target cell by recognition of cell surface receptors and kill the cell via diverse mechanisms. Bacteriocins produced by Gram-negative bacteria can be divided into three categories based on size. The most studied SOS-induced colicin-like bacteriocins (30-80 kDa) kill target cells by pore formation, nuclease activity or cell wall disruption (Chavan and Riley, 2007). Microcins, which are smaller than 10 kDa, are mostly produced in response to stress and poor nutrient conditions; these toxins are non-SOS-inducible, display various mechanisms of uptake, are produced by several biosynthetic pathways and might undergo posttranslational modifications (Rebuffat, 2011). Last, phage tail-like bacteriocins have been proposed to be defective phages or to have originated from phages that evolved to function as bacteriocins; these toxins are nuclease- and protease-resistant and kill sensitive cells by depolarization of the cell membrane (Chavan and Riley, 2007). Bacteriocins ensure improved survival and virulence abilities, and thus overall better ecological fitness of the producer strain as a consequence of the affected bacterial population dynamics (Riley and Wertz, 2002).

Although little is known about bacteriocins produced by plant pathogenic bacteria, *Pectobacterium carotovorum* strains have been studied and appear to harbor genes encoding peptide bacteriocins. In particular, carocin S1, a low-molecular weight bacteriocin isolated from the *P. carotovorum* subsp. *carotovorum* strain, appears to be secreted by T3SS (Chan *et al.*, 2009), possesses nuclease activity and is induced by glucose, lactose and SOS agents (Chuang *et al.*, 2007). Carocin S2 is a colicin-like bacteriocin with ribonuclease activity produced by *P. carotovorum* that is induced by ultraviolet radiation (Chan *et al.*, 2011). Carocin D, produced by 5 strains of *P. carotovorum* subsp. *carotovorum,* is similar to colicin and has DNase activity (Roh *et al.*, 2010). Carotovoricin Era and carotovoricin Erb are phage-tail bacteriocins 68 kDa and 76 kDa in length, respectively, with different *P. carotovorum* subsp. *carotovorum* strain specificities (Nguyen *et al.*, 2001). The colicin-like bacteriocins pectocin M1 and pectocin M2 target some *P. carotovorum* and *P. atrosepticum* strains with increased activity under iron-limiting conditions (Grinter *et al.*, 2012). However, to date no evidence of bacteriocins produced by *Dickeya* spp. has been reported. Because bacteriocins are responsible for the inhibition of a narrow spectrum of bacteria, their use in agricultural practices represents an attractive approach to control the spread of targeted bacterial species and may therefore be developed as new strategies for plant disease control (Montesinos, 2007).

1.8. From pre- to post-genomics of soft rot enterobacteria

Nucleic acid assays to investigate microbial communities represent pre-genomic tools. Several approaches have been applied to define phylogenetic relationships in the past decades. DNA-DNA hybridization (DDH) applied to plant pathogenic enterobacteria for the first time in 1970s (De Ley *et al.*, 1970; Brenner *et al.*, 1972) has been defined as gold standard method for species delineation (Stackebrandt *et al.*, 2002 (Stackebrandt *et al.*, 2002). In DDH, pairs of highly purified single-stranded DNAs are hybridized to estimate DNA similarity. The thermal denaturation midpoint and quantification of hybrid formation between two single-stranded DNAs are used to describe a species; for example, 70% DDH and a thermal denaturation midpoint less than 5˚C depicts strains belonging to the same bacterial species (Wayne *et al.*, 1987). Since DDH was time consuming and limited to pairwise comparison, phylogenetic analysis of amino acid or nucleotide sequences of conserved molecules have been widely used to infer species delineation in bacterial taxonomy (Woese, 1987). Particularly, the small subunit ribosomal RNA (16S RNA) has become a standard for determining phylogenetic relationships in the pre-genomic era because it is present in all organisms and possessing conserved functions and contains both conserved and variable sequences (Peter and Young, 1994). However, in several cases, 16S RNA analysis was not able to distinguish closely related species due to its low resolution. Several alternative phylogenetic methods has been proposed, including multilocus sequence analysis (MLSA), which is performed by comparing concatenated sets of housekeeping gene sequences (Gevers *et al.*, 2005).

Since the development of the high-throughput pyro-sequencing method, a large amount of highly informative genome sequence data has been obtained due to the reduction in sequencing costs (Margulies *et al.*, 2005); simultaneously, an array of computational and functional genomic tools have been developed. For instance, average nucleotide identity (ANI) (Konstantinidis and Tiedje, 2005) allows the measurement of phylogenetic relationships between two genomes on the basis of nucleotide identities within shared genes. All of this sequencing data allows the clarification of the taxonomic position of many plant pathogenic bacterial species, thereby allowing the detection and quantification of genetic diversity in much finer detail. An example of a revised phylogeny based on genomic sequence analysis is represented by *P. wasabiae* 3193, which was previously misclassified as *P. carotovorum* (Nykyri *et al.*, 2012). In 2004, the report of the first genome sequence of a plant pathogenic enterobacteria (*P. atrosepticum* SCRI1043) (Bell *et al.*, 2004) marked the beginning of the post-genomic era for soft rot enterobacteria. Today, the availability of 39 *Dickeya* and 45 *Pectobacterium* genome sequences in the NCBI database represents a valuable source of genomic information and enables an array of applications.

If genomics provides the genome sequence of an organism and can reveal new information about basic biochemical and cellular functions, post-genomic applications allow the exploitation of whole genomes by making comparisons and highlighting the flexibility of genome organization. In

particular, a genome comparison of soft rot enterobacteria not only shows shared pathogenicity and lifestyle mechanisms but also reveals pathogen-specific features that are required for the colonization of new ecological niches and the successful emergence of the pathogens.

Pathogenicity and virulence factors and transcriptional regulators often allow bacteria to instantaneously adapt to new, fast changing environments or hosts and are often found within mobile genetic elements. Bacteria can acquire new genes and functions residing in mobile genetic elements from other microbes through horizontal gene transfer (HGT). HGT of pathogenicity islands (PIs) might indeed be the means by which the enterobacteria *Dickeya* and *Pectobacterium* switched niches (Toth *et al.*, 2006) and evolved to become plant pathogens. However, to ensure their dispersal and survival, these bacteria maintained their non-pathogenic relationships with potential vectors and hosts such as herbivores and plant-associated insects (Basset *et al.*, 2003). As more bacterial genome sequences become available, comparative genomics will enable researchers to highlight the precise molecular processes that drive the evolution of a plant pathogen and favor its successful emergence in wider environmental niches. To date, the NCBI database contains several *Dickeya* genomes, including ten *D. zeae,* seven *D. solani*, six *D. chrysanthemi,* six unclassified *Dickeya* species, four *D. dadantii*, four *D. dianthicola*, and two *D. paradisiaca*. The availability of multiple *Dickeya* genome sequences and their annotations enables comparisons between different species by computational approaches, thereby identifying new features that may be important factors for pathogen evolution. A genome comparison between *D. solani* and the closely related species *D. dadantii* revealed that the *D. solani* genome harbored toxins and NRPS/PKS-encoding genes that played important roles in bacteria-bacteria interactions and might have facilitated the establishment of *D. solani* in the potato (Pedron *et al.*, 2014). Moreover, the *Dickeya* genomic dataset represents a solid base of information that can be used to perform functional genomic studies.

1.9. Phenotypic and virulence characterization of *Dickeya* strains isolated in Finland

Several *Dickeya* strains were isolated from potato stems, tubers and river water in a 2004-2005 survey in Finland. Phylogenetic analysis of 16S and 16S–23S rDNA sequences of Finnish *Dickeya* isolates grouped the strains into three clusters: one cluster corresponding to *D. dianthicola* and two other clusters not resembling any of the previously characterized *Dickeya* spp. (Laurila *et al.*, 2008).

Biochemical tests were performed to identify the *Dickeya* isolates; the results showed different biochemical profiles for each cluster. Strains in the cluster that was phylogenetically similar to *D. dianthicola* were identified as biovars 1 and 7, which supported the conclusion that these strains belonged to *D. dianthicola* species. Strains isolated from river water that clustered together into an unknown group had a biochemical profile that did not match any characterized biovar and were later

identified as belonging to the new species *D. acquatica* (Parkinson *et al.*, 2014). Unknown *Dickeya* strains from the third cluster were isolated from potato tubers and stems and grew on arabinose, melibiose, raffinose and mannitol but did not utilize tartrate, were negative in the inulin test and were unable to hydrolyze arginine under anaerobic conditions. They did not grow at 39°C in liquid medium, but formed small colonies at 39°C on solid nutrient agar medium (I). This biochemical profile was identical to biovar 3 (Samson *et al.*, 1987; Ngwira and Samson, 1990); thus, these strains resembled new *Dickeya* strains isolated in the Netherlands (Slawiak *et al.*, 2009). Although other biovar 3 *Dickeya* strains previously identified on potatoes in Australia and Peru and in ornamentals and corn in Europe (Cother and Powell, 1983; Ferreira-Pinto *et al.*, 1994; Nassar *et al.*, 1994) were included in the species *D. dadantii* and *D. zeae*, the new biovar 3 strains isolated in Finland and the Netherlands appeared to be phylogenetically different (Laurila *et al*., 2008). Strains belonging to this new unidentified *Dickeya* clade were isolated in Finland from primarily newly released potato varieties for which the seed potatoes was recently imported, supporting the hypothesis that these strains might have spread from ornamentals to potatoes in Continental Europe and moved to different countries, including Finland, via the seed potato trade (Toth *et al.*, 2011).

To characterize the new *Dickeya* strains, their virulence was evaluated in a field trial with potato plants grown from vacuum-inoculated seed tubers. All strains were able to cause stem rot; half of the strains caused the non-emergence of potato plants and most strains delayed the growth of a few potato plants. Although rotting of the plants appeared similar regardless of the inoculated *Dickeya* strain, the blackleg symptoms differed from those caused by *P. atrosepticum* and a considerable variation in disease progress was recorded between plants inoculated with different *Dickeya* strains. The first symptom observed was wilting; some wilted plants also exhibited blackleg-like rotting of the stem base. Later, the infection spread up to the pith of the stem, resulting in decay of the leaves and petiols. The stem bases of many plants stayed green and appeared to be asymptomatic for a long time and were the last parts of the plant to rot (Laurila *et al.*, 2010). This internal hidden *Dickeya* stem decay represents a high risk that the incidence of *Dickeya* symptoms is ignored or underestimated during field inspections.

D. dianthicola and the new biovar 3 strains appeared to be the most virulent based on the disease symptoms reported (Laurila et al., 2010). Their infections also notably reduced potato tuber yields and increased the proportion of rotten tubers during harvest and storage. In general, a proportional relationship between the incidence of stem rot and the total proportion of rotten tubers at harvest and in storage was found, suggesting that contamination of seed tubers with *Dickeya* may lead to economic losses at all steps of the potato production chain.

The yield reduction and high virulence of *D. dianthicola* and some of the newly identified biovar 3 strains addressed the need for additional studies on the ecology and pathogenesis of these potato pathogens to reduce their negative economic impact and develop disease management strategies.

2. AIM OF THE STUDY

In this thesis, the new bacterium *D. solani* that infects potato plants and tubers has been characterized. First, a real-time PCR test was developed for its diagnosis; then, the taxonomy of the bacterium was analyzed in a European-wide collaboration and the pathogen was verified to be a new species. The genome of a Finnish isolate was sequenced, and genomic approaches were applied to analyze the genome content and investigate gene functions. The information provided by the genomic research enabled us to utilize post-genomic approaches focused on the molecular characterization of bacterial competition *in vitro* and inside the host plant *in vivo* to identify the possible mechanism underlying the fast spread of the pathogen. The last section of the dissertation focused on the investigation of gene clusters that might enable *D. solani* to take over as a potato pathogen and overcome other soft rot potato pathogens during host infection.

The whole study can be divided into four parts:

1. Development of a real-time PCR test to analyze the amount of *D. solani* in plant tissue.
2. Assessment of the taxonomy of *Dickeya* biovar 3 bacterial isolates and description of *Dickeya solani* sp. nov..
3. *D. solani* D s0432-1 genome sequencing, comparative genomics and identification of a new combination of gene clusters encoding for secondary metabolites.
4. Experimental characterization of a *D. solani* antibacterial compound affecting bacterial competition.

3. SUMMARY OF MATERIALS AND METHODS

The methodologies used in this study are listed in Table 1, and a detailed description of each method is provided in the original papers.

Table 1. Summary of the methods used in this study.

Method	**Publication**
Bacterial strains cultivation	I-II-III-IV
Bacterial DNA extraction	II-III-IV
Primer design	I-II-III-IV
Molecular cloning	II-IV
Plasmid constructs	II-IV
PCR	I-II-III-IV
Real-Time PCR	I
Transposon mediated mutagenesis	IV
Bacterial inhibition assay *in vitro* and *in vivo*	IV
In vitro bacterial growth curves	I-IV
Enzymatic assays	I-IV
Virulence assay in field condition	I
Potato tuber inoculation	I-IV
Comparative genomics	III-IV
Genome browsing	III-IV
Genomic island prediction	III
Open reading frame prediction and annotation	III-IV
Protein function prediction	III-IV
Sequence alignment	II-III-IV

4. SUMMARY OF RESULTS AND DISCUSSION

4.1. Development of a real-time PCR method for the detection of *Dickeya* species from potato tissue

To verify that the disease symptoms observed on plants from the field inoculation test conducted in Finland were caused by *Dickeya* species, bacterial colonies in the plant tissues were isolated on semi-selective CVP medium (I). The bacteria were identified as *Dickeya* species by PCR using primers ADE1 and ADE2 that are specific for *Erwinia chrysanthemi* species (Nassar *et al.*, 1996). The detection of *Dickeya* species required an initial isolation and culture of bacteria followed by a conventional PCR, which was a laborious method. Thus, a real-time PCR test performed directly from the tissue extracts was developed.

Three real-time primer pairs were developed at the Tataa Biocenter in Gothenburg (Sweden) using the Allele ID software. These primer pairs were designed to amplify sequences between 16S and 23S DNA of *Pectobacterium* and *Dickeya* species and to exclude recognition of other microbes commonly present in the potato and soil environment, such as *Klebsiella*, *Enterobacter*, *Xanthomonas*, *Paenibacillus*, *Bacillus*, *Ralstonia*, *Pseudomonas* and *Clavibacter* strains. Primers Dr (TTTCACCCACCGTCAGTC) and Df (AGAGTCAAAAGCGTCTTG) amplify a 133 bp sequence of *D. dianthicola* and biovar 3 strains. Moreover, the Dr and Df primer pair can amplify the same fragment from other *Dickeya* species type strains, but do not anneal with *D. aquatica* strains and *Pectobacterium* species. A second primer pair (forward: AAGTGCTCACACAGATTG and reverse: GTTACAGCCGTGAAAGGG) was developed to recognize only *P. atrosepticum* and *P. carotovorum* strains. Finally, a third primer pair (forward: AAAAGCGTCTTGCGAAGC and reverse: TCCCTGACCGTGACTTTC) recognized only *D. aquatica* strains, but this pair has not been tested.

To determine the optimal real-time PCR conditions, the *Dickeya* and *Pectobacterium* primer pairs were tested first in conventional PCR and later in real-time PCR with different settings. A temperature gradient between 48˚C and 62.5˚C was used in the conventional PCR with bacterial genomic DNA to determine the optimal annealing temperature (Ta). A Ta of 60˚C was chosen for the real-time PCR reactions because it provided more specific amplification. Three primer concentrations (0.2 µM, 0.4 µM and 0.6 µM) were also tested in conventional PCR; the highest concentration was chosen for the real-time PCR. Because the amplified fragment of *Dickeya* species has a high GC content, 2.5% DSMO was tested in the real-time PCR reaction. A concentration of 0,4% BSA was also tested; however, neither additive contributed to the enhancement of the real-time PCR reaction. Different concentrations of bacterial genomic DNA and different dilutions of plant extracts from diseased and artificially inoculated tubers and pure cultures of bacterial strains were used as the templates for the real-time PCR reactions. The optimal template volume was set at 5 µl and the plant

extract dilution at 1/100. Derivative plots of melting curves suggested high specificity of the primer pairs for the amplified PCR product using plant extracts, genomic DNA and bacterial cultures as templates. Furthermore, no primer dimer formation was visible because no additional peaks appeared in addition to the specific product at approximately 82.5˚C. The real-time PCR assay could detect bacterial cells even without DNA isolation and when a low number of target cells was present. However, standard curves made with 10-fold serial dilutions of overnight LB cultures showed high Ct values for templates containing 10^2 or less bacterial cells per ml. Moreover, differences in Ct values between samples with bacterial contents lower than 10^2 cells/ml were not easily detectable, and their Ct values were too close to the negative water control. Due to this lack of sensitivity when using very low numbers of cells and to avoid misannealing of the primers due to the scarcity of bacterial target DNA and abundance of plant DNA when using this method for diagnostic purposes, only samples with Ct values corresponding to more than 10^2 bacteria per ml were considered to be clearly positive.

This real-time PCR method was applied to detect bacteria from the same diseased stem samples previously analyzed by culture and conventional PCR (I). Potato plant tissue extract was used directly in the real-time PCR reaction. The presence of *D. dianthicola* and biovar 3 strains was confirmed using primers Df and Dr because the results were identical to the previous PCR analysis with primers ADE1 and ADE2 (I). Furthermore, the primer pair Df and Dr was used to analyze the *Dickeya* content in various samples collected from fields in Finland within the Euphresco project "Assessment of *Dickeya* and *Pectobacterium* spp. on potatoes and ornamentals".

Over 200 samples, including potato stems and tubers and weeds growing near infected potato fields and water courses, were investigated for the presence of *Dickeya* contamination (unpublished data). Most potato samples originating from recently imported cultivars (some representing the highest seed classes) were found to be positive with the test. However, cultivars with a Finnish origin also tested positive in two cases, suggesting the spread of *Dickeya* from imported material into other cultivars in Finland. Furthermore, potato stem samples with dark and pale rot and rot in various parts of the stem tested positive. Specifically, plants showing green stem bases but exhibiting rot in the upper parts of the stem (as shown in Figure 3) were positive in the test, suggesting that the blackleg symptoms caused by some *Dickeya* species often differ by the ones caused by *Pectobacterium* species. Positive test results were also obtained from voluntary potatoes, but not from the analyzed weed samples.

The newly developed real-time PCR with primers Df and Dr not only allows the recognition of biovar 3 *Dickeya* but also detects all known *Dickeya* species (with the exception of *D. aquatica*). Thus, this method represents a useful diagnostic tool for the fast identification of *Dickeya* strains from symptomatic plants. The ability to perform the real-time PCR essay directly on tissue extracts bypasses laborious and time-consuming practices such as bacterial culture and genomic DNA extraction.

4.2. Recognition of *D. solani* as a novel species

New *Dickeya* isolates reported from Finland and elsewhere in Europe since 2004 were identified as biovar 3 (Toth *et al.*, 2011). Although some *D. dadantii* and *D. zeae* strains were also biovar 3, the new *Dickeya* isolates clustered together in a different clade based on the phylogenetic analysis of the 16S-23S IGS (Laurila *et al.*, 2008), *dnaX* (Slawiak *et al.*, 2009) and *recA* (Parkinson *et al.*, 2009) genes and were distinct from other *Dickeya* species by REP-PCR (Slawiak *et al.*, 2009). Because the new biovar 3 clade has become the principal potato pathogen in Europe in a short period of time and dominates the previously known *Dickeya* and *Pectobacterium* pathogens (Toth *et al.*, 2011), its taxonomic position within the genus *Dickeya* needed to be clarified. The tentative name *Dickeya solani* was proposed.

To classify the new biovar 3 strains, the 16S-23S rDNA IGS of 34 *Dickeya* strains belonging to different species were sequenced. The IGS sequences of eleven biovar 3 strains from potatoes isolated in the Netherlands, Finland, Poland, United Kingdom, France and Israel and a similar strain isolated from hyacinth in the Netherlands were analyzed and compared to other *Dickeya* reference strains. Among the biovar 3 strains, D t042 (PRI 3294) and D s0432-1 (PRI 3295) were isolated from a Finnish potato tuber and stem, respectively, during the survey in 2004/2005, and their phenotype and virulence were previously characterized (I). The PCR reactions were performed as described by Fessehaie *et al.* (2002) (Fessehaie *et al.*, 2002) using the universal DNA primers 1491f and L1r. PCR fragments for sequencing were prepared from the smaller of the two PCR fragments (~450 bp) by purification of the bands. After DNA sequencing, both sequence strands were assembled into a single contig and the 16S rDNA and 23S rDNA portions were manually removed at the 5' and 3' ends, respectively. The IGSs were aligned using the ClustalX2 multiple sequence alignment program (Larkin *et al.*, 2007), and a phylogenetic tree was generated using the maximum-likelihood method with the MEGA6 software (Tamura *et al.*, 2013) (Figure 4).

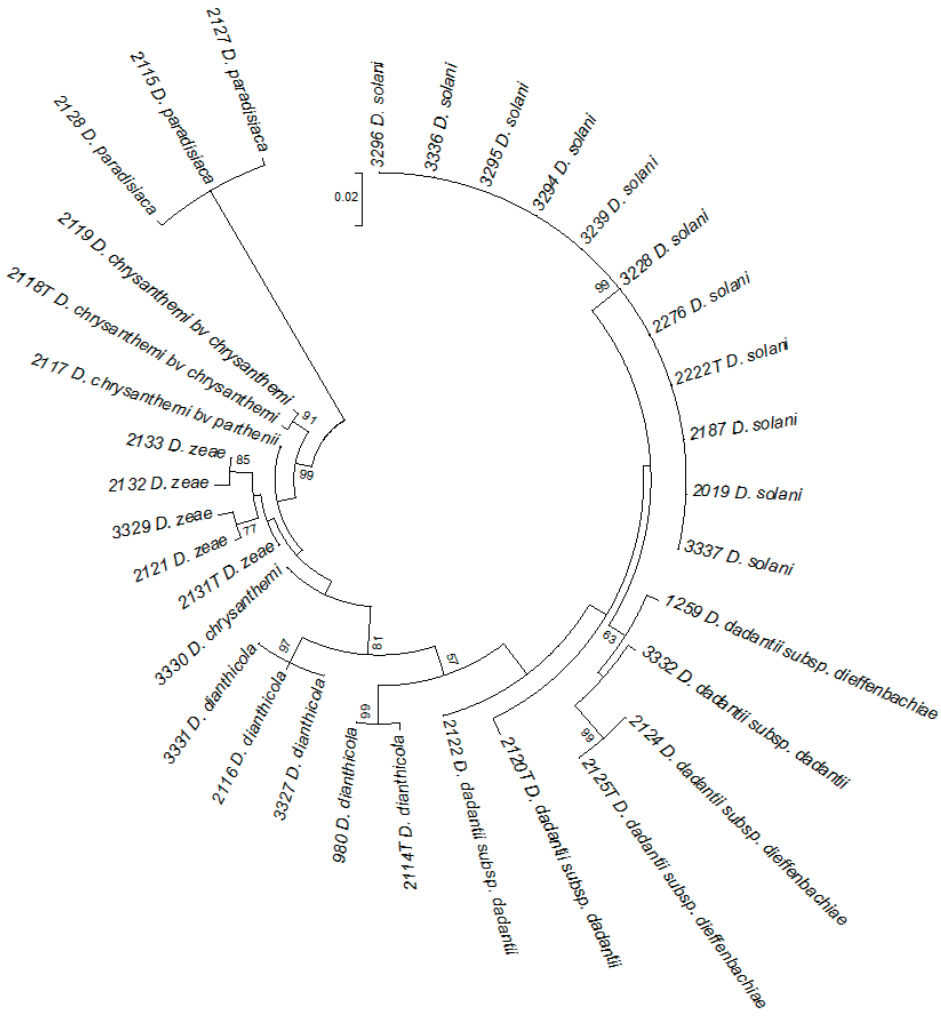

Figure 4. Phylogenetic tree of IGS sequences (343 bp) of 34 *Dickeya* strains belonging to different species. Gaps or missing data in the alignment were excluded when the site coverage was below 95 %. Bootstrap percentages were calculated with 1000 replicates, and the Nearest-Neighbor-Interchange was used as a heuristic method to improve the likelihood of the tree.

All strains belonging to the same *Dickeya* species clustered together in the phylogenetic tree based on IGS sequencing. Additionally, eleven strains of *D. solani* formed a distinct clade that was separated from the other five *Dickeya* species. Twenty-seven of these IGS sequences were used within a European collaboration to classify the new biovar 3 strains. Other partners provided the sequences of other housekeeping genes (*dnaN*, *fusA*, *gapA*, *purA*, *rplB*, *recA*, *dnaX*, *rpoS* and *gyrA*) from the same *Dickeya* strains selected by the European collaboration. Phylogenetic analysis of the sequence alignments was performed for each housekeeping gene and the 3520 bp sequence containing the 10 concatenated genes. Phylogenetic trees of single gene alignments showed that the *D. solani* strains were similar or identical (II). Multilocus sequence analysis using all of the

previous gene sequences concatenated from 27 *Dickeya* strains not only clustered the *D. solani* strains together but also confirmed that they were most closely related to *D. dadantii* subsp. *dadantii* and *D. dadantii* subsp. *dieffenbachiae* (Figure 5).

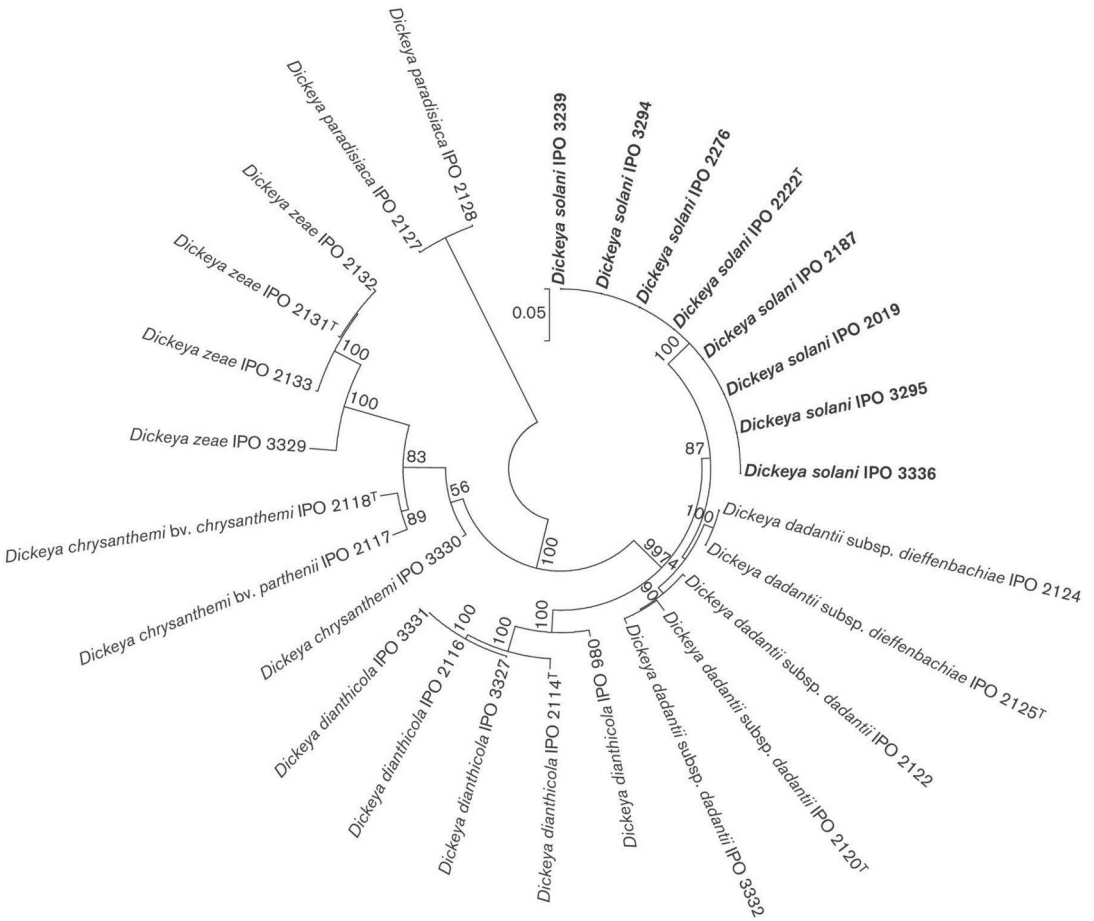

Figure 5. Phylogenetic tree of concatenated sequences of *dnaX*, *dnaN*, *fusA*, *gapA*, *gyrA*, *purA*, *rplB*, *rpoS*, *recA* and IGS from 27 *Dickeya* strains (II).

Other phenotypic and molecular characterization methods, such as MALDI-TOF mass spectrometry (MS), pulsed field gel electrophoresis (PFGE) after restriction enzyme digestion, DNA-DNA hybridization and ANI, were performed by collaborators on the 27 *Dickeya* strains and confirmed that the new biovar 3 isolates represented a novel species named *Dickeya solani* that was closely related to *D. dadantii* (II). Moreover, the *D. solani* strains isolated from different countries and two hosts were homogeneously grouped together based on a phylogenetic analysis of the MLSA and pulsed field gel electrophoresis data, which suggested a high degree of clonality.

4.3. Genomic approach to taxonomy and virulence of the *D. solani* D s0432-1 strain

A genomic approach was used to elucidate the emergence of *D. solani* by studying the differences between this new species and the other soft rot pathogens. *D. solani* strain D s0432-1 isolated in Finland from a potato stem was previously phenotypically characterized as a biovar 3 *Dickeya* in the first paper (I) and was renamed IPO 3295 and confirmed to belong to the novel species *D. solani* in the second paper (II).

4.3.1. *D. solani* D s0432-1 genome sequencing and comparative genomics

The genome of strain D s0432-1 was sequenced; the total length was 4.9 Mb, including three gaps that could not be filled. The whole genome sequence (WGS) of *D. solani* D s0432-1 is deposited in GenBank (accession number AMWE00000000) and is composed of four sequences (AMWE00000001, AMWE00000002, AMWE00000003 and AMWE00000004). The names of the contigs and locus tags mentioned below lack the first part of their codes (AMWE0000000 and A544, respectively) for simplicity.

BLAST analysis of the DNA regions flanking the gaps revealed that the first gap contained domains similar to the multi repeat-anchored surface protein (between contig 1 and contig 2), while the second (between contig 2 and contig 3) and third (between contig 3 and contig 4) gaps contained genes involved in contact-dependent growth inhibition based on the fact that their terminal sequences contained hemagglutinin repeat domains and hemagglutination activity domains, respectively. The ORF flanking the third gap on the fourth contig was annotated as the *cdiB* gene (Aoki *et al.*, 2010). Syntheny analysis performed with Mauve (Darling *et al.*, 2010) revealed that the lowest number of gene rearrangements was found between *D. solani* and *D. dadantii* 3937. Furthermore, ANI calculated with different algorithms among five *Dickeya* species and *P. atrosepticum* was highest between *D. solani* D s0432-1 and *D. dadantii* 3937. The ANI value of this pairwise comparison was 0.94, which was identical to the result obtained during the analysis of *D. solani* species delineation (II). This value is below the threshold for species delineation (Richter and Rossello-Mora, 2009), indicating that *D. solani* D s0432-1 and *D. dadantii* 3937 should be considered as two different species despite the fact that they share high sequence similarity.

The *D. solani* D s0432-1 genome sequence contains 4173 protein coding genes. Manual identification of known virulence determinants revealed that it harbored several types of PCWDEs (including pectinases, cellulases and proteases typical of soft rot enterobacteria), as well as genes involved in the uptake and catabolism of pectic substrates. Moreover, all of the virulence determinants previously demonstrated to be encoded by other *Dickeya* species were also found in *D. solani* D s0432-1 (III, Table S1).

A nucleotide BLAST was performed between *D. solani* D s0432-1 and nine other sequenced strains (including *Dickeya* and *Pectobacterium* species); the results are shown in the circle graphic in figure 6. Although *D. solani* D s0432-1 shares large portions of its genome with *D. dadantii* 3937 and other *Dickeya* species, some parts are shared only with one or a few *Dickeya* species or are not shared at all. To investigate the peculiarities of *D. solani*, the genomic regions that were less shared with the other species were studied in greater detail.

4.3.2. Unique combination of large gene clusters in *D. solani* D s0432-1

Gene syntheny in region 1, comprised between ORF2708 and ORF2719 in the fourth contig of the D s0432-1 genome, was conserved between D s0432-1 and *D. zeae* Ech586. This region exhibited protein identity only with the *D. zeae* Ech586 strain and was not found in any other soft rot bacteria. Some of the genes were also found in an organism called *Teredinibacter turnerae*, an endosymbiont of marine wood-boring shipworms, but the protein identity was much lower. The 12 protein coding genes in *D. solani* region 1 included annotations for trans-AT polyketide synthases (PKS), non-ribosomal peptide synthases (NRPS) and amino acid adenylation domain proteins, indicating the presence of a PKS/NRPS hybrid system. PKS and NRPS modules generally produce molecules with antibiotic and phytotoxic activities and are also responsible for the increased virulence in some bacterial plant pathogens (Donadio *et al.*, 2007). However, the structure and function of the molecule produced by this cluster in *D. solani* and *D. zeae* Ech586 remain unknown.

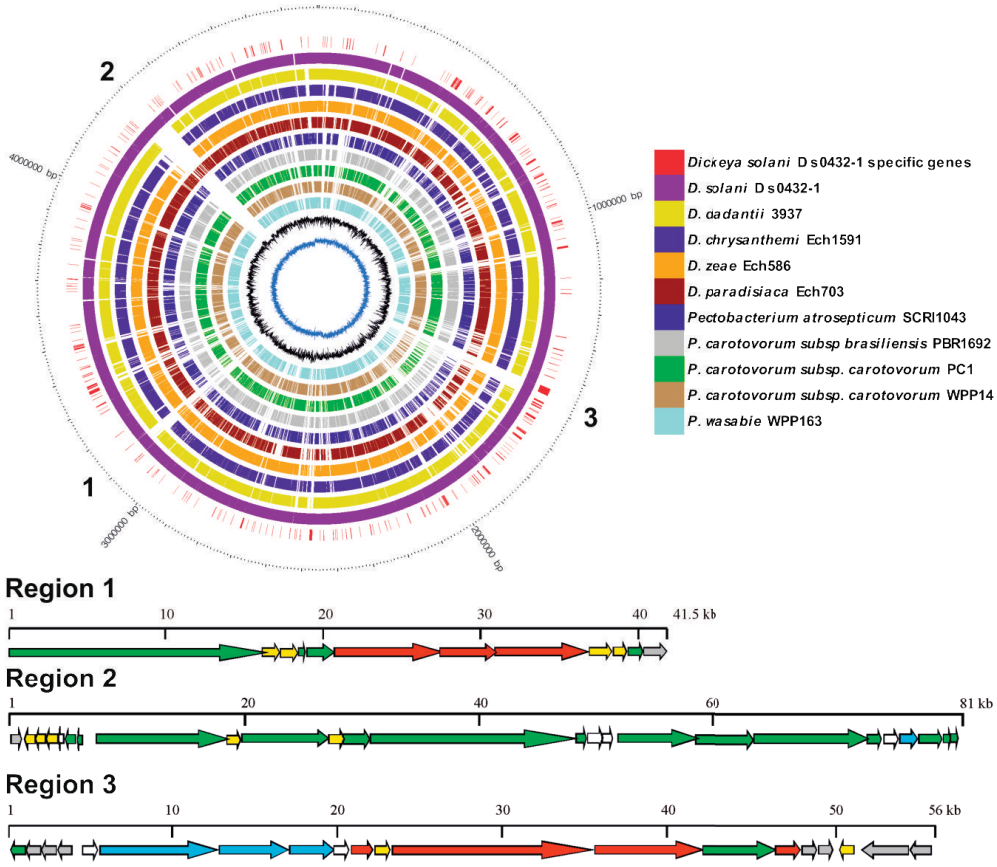

Figure 6. Comparative genomics between *Dickeya* strains. Predicted open reading frames specific to *D. solani* D s0432-1 are represented as the outermost red ring. In the comparison of gene coding sequences, three large genomic regions are shared between only a few *Dickeya* strains. The three genomic regions from *D. solani* D s0432-1 code for non-ribosomal peptide synthetases and polyketide synthases. In the D s0432-1 clusters, the ORFs are marked with different colors based on their function: red for NRPS, green for PKS, blue for FAS-like, grey for transport, yellow for tailoring and white for hypothetical.

Region 2 of *D. solani* (ORF3690 and ORF3714, fourth contig) is composed by 25 ORFs, among which 13 ORFs encoded for PKS modules, acyl carrier proteins and other genes related to the production of secondary metabolites. Similar clusters showing the same gene organization were found in *S. marcenscens* and *S. plymuthica* strains and encoded for a trans-AT PKS involved in the production of the antifungal and anti-oomycete molecule Oocydin A (Matilla *et al.*, 2012). This region is also present in the *Serratia odorifera* 4Rx13 (GenBank accession number CP006250), a strain isolated from the potato rhizosphere. This homology suggests that *D. solani* D s0432-1 might be able to synthetize Oocydin A and that it might have acquired this cluster from other soil bacteria by lateral

transmission. Among other *Dickeya* spp., *D. paradisiaca* (Ech703 strain) is the only species showing a similar gene clusters (Figure 6).

A third interesting region in the *D. solani* genome (ORF1389-ORF1408 second contig) contains genes showing fatty acid synthase (FAS), PKS and NRPS domains, and was not detected in any *Dickeya* and *Pectobacterium* genomes available so far (Figure 6). It showed partial similarity with the gene clusters in the soil bacterial strains *Serratia plymuthica* AS12, and A30. Moreover, *D. solani* ORF1394 showed similarity to the 5′ end of *zmsA* from *D. zeae* DZ1 (a gene involved in the production of zeamine). A genomic cluster similar to the one containing *zmsA* in *D. zeae* DZ1 has been found in *S. plymuthica* RVH1 leading to the biosynthesis of zeamine. This molecule has been shown to have antibiotic and phytotoxic activities. Although a direct sequence comparison was not possible due to the lack of sequencing information for the zeamine cluster of *D. zeae* DZ1, it is very likely that *D. solani* might be able to produce a molecule similar to zeamine. Furthermore, the absence of this cluster from the other sequenced *Dickeya* and *Pectobacterium* genomes suggests that *D. solani* may have acquired it from the *D. zeae* strain or other soil bacteria.

The horizontal gene transfer hypothesis is also supported by the fact that *D. solani* regions 2 and 3 are predicted to be located on two distinct genomic islands. In contrast to most of the other genomic islands detected in *D. solani* that also appeared in its closest relative *D. dadanti* 3937, these two regions seemed to have been acquired more recently because they were absent from *D. dadantii* 3937.

These three large genomic areas represent specific features of *Dickeya* solani that are not commonly shared by other *Dickeya* and *Pectobacterium* species. Moreover, the combination of the three gene clusters has not been found in any other organism. The fact that all three regions are suggested to encode for secondary metabolites indicates that *D. solani* harbors a unique combination of genes that produce molecules that may be toxic to microbes and plants.

4.3.3. Genes specific to *D. solani* D s0432-1

In addition to the distinctive secondary metabolite gene clusters, *D. solani* harbored a set of single genes that were not found in any other *Dickeya* and *Pectobacterium* strains when compared with the Blast algorithm. Genes coding for multidrug efflux systems were specific for *D. solani*. One of these transporters is located adjacent to the PKS/NRPS cluster in region 1, while the ABC-type transport system is part of the same predicted genomic island as the FAS/PKS/NRPS cluster in region 3. These localizations suggest that these transport systems might be involved in the secretion of secondary metabolites produced by the adjacent gene clusters (Gaisser and Hughes, 1997). A third specific multidrug efflux system, of Resistance-nodulation-division (RND) type, is specific to *D. solani*; its genes fall into a predicted genomic island, suggesting also their lateral acquisition. RND transporters pump a broad range of toxic molecules directly into the external medium. Thus, this

transporter might confer *D. solani* with increased resistance against inhibitors compared to the other *Dickeya* and *Pectobacterium* species lacking these transporters, thereby allowing survival and improved defense mechanisms in new environments (Nikaido, 2009). Two groups of genes encoding type I restriction-modification (R-M) systems were also lacking from the genomes of the other soft rot enterobacteria analyzed. However, one of them is part of a predicted genomic island and shows high protein identity with *P. wasabiae* SCC3193. The presence of these R-M systems might protect *D. solani* from the acquisition of potentially dangerous DNA (i.e., bacteriophages), thereby representing an advantage during the colonization of new ecological niches (Blumenthal and Cheng, 2002). Five ORFs specific to *D. solani* were related to the production of demethylmenaquinone methyltransferase, an enzyme that catalyzes the synthesis of menaquinone that is an electron carrier involved in anaerobic respiration. This enzyme is also present in bacterial and fungal isolates that interact with plants and ensures microbial growth under microaerobic conditions (Xie *et al.*, 2011). A sixth demethylmenaquinone methyltransferase gene is present in *D. solani* and is also conserved in other *Dickeya* species, but the five additional genes present in *D. solani* might alter its behavior under anaerobic conditions. Other *D. solani* specific genes are involved in the degradation of peptides, amino acids and organic molecules. Furthermore, *D. solani*-specific regulators and repressors were found spread throughout the genome; these genes might enhance the adaptation of *D. solani* to new environments or hosts by affecting virulence gene expression.

4.4. Postgenomic approach to detect toxic compounds produced by *D. solani*

The finding of a unique combination of clusters in the *D. solani* genome that are possibly involved in the production of toxic secondary metabolites might explain its invasive character. Toxic compounds produced by *D. solani* and absent from the other *Dickeya* and *Pectobacterium* species might confer *D. solani* with the ability to overcome competitors during host plant colonization, ensuring better fitness. To determine if toxic compounds are produced as well as to study their activity and spectrum of action, bacterial competition between *D. solani* and other common bacterial potato soft rot pathogens was investigated.

4.4.1. *D. solani* inhibits the growth of *Pectobacterium* strains

A bacterial inhibition essay was performed using the agar diffusion technique in Minimal Inducing Medium (MIM) using three strains of *D. solani*, including the Finnish strain D s0432-1. An inhibition halo was present around *D. solani* point inoculated colonies growing with *P. carotovorum* subsp. *carotovorum* SCC1 and *P. atrosepticum* SCRI 1043 (Figure 7), indicating the ability of *D. solani* isolates to produce a molecule toxic to some closely related bacteria. However, *P. wasabiae* SCC3193 was not susceptible to the growth inhibition by *D. solani*.

To detect the genes responsible for the production of the toxic molecule, transposon insertion mutagenesis was performed on *D. solani* D s0432-1. The mutant pool was screened in an inhibition assay in competition with the SCC1 and SCRI 1043 strains to find mutants exhibiting a lack of inhibition activity. Three mutants were selected and the insertion points of the transposon were detected due to the availability of the D s0432-1 sequenced genome; notably, all of the insertion points fell within the same gene cluster. Interestingly, the mutated gene cluster was not one of the previously described PKS/NRPS clusters (regions 1, 2 and 3 in the D s0432-1 genome, Figure 6) proposed to be involved in the production of bacterial toxins. The mutated operon consists of 14 genes (from ORF0999 to ORFs1010), and in addition to the previous annotation was shown to include two new ORFs (apORF1 and apORF2) (Figure 7). A large portion of this cluster from ORF1002 to ORF1010 was computationally predicted to encode a bacteriocin with an unknown structure. Conserved domain analysis revealed that the *D. solani* bacteriocin cluster contains domains found in protein families involved in amino acid conversions and antimicrobial compound synthesis. The presence of these domains in the biosynthesis genes suggested that the toxic product of the *D. solani* cluster might be a modified small peptide or an amino acid derivate. Furthermore, high similarity in the domain architecture was found with Microcin C7 of *E. coli*, indicating that a similar compound might be produced by *D. solani* strains. One of the mutants (strain M1001 that had its wild type gene disrupted by a transposon insertion) was complemented with the full-length gene. This led to the re-appearance of the inhibition halo, indicating that the mutated gene cluster was involved in the growth inhibition of *Pectobacterium* strains (IV).

4.4.2. Growth inhibition activity in other *Dickeya* species

Comparative analysis of nucleotide sequences of the *D. solani* gene cluster confirmed the presence of the same bacteriocin operon (99% nucleotide identity) in sequenced *D. solani* strain IPO2222, showing only two single insertions.

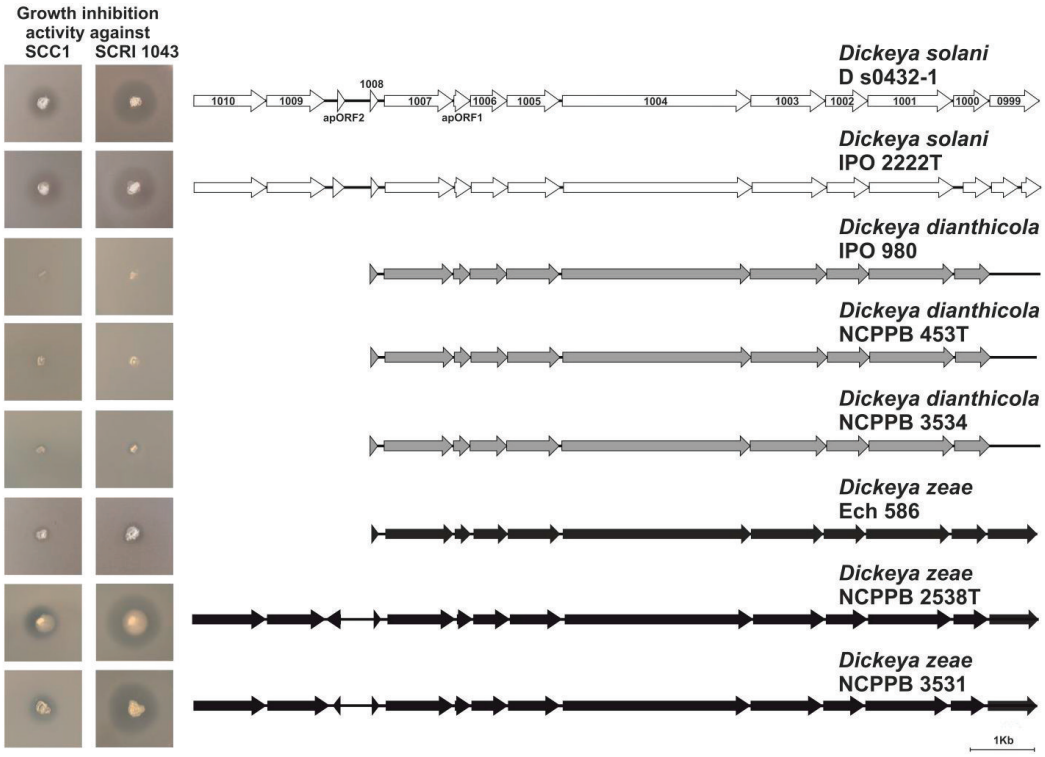

Figure 7. Comparison of the bacteriocin gene clusters present in three *Dickeya* species. Growth inhibition activity for each *Dickeya* strain against *P. carotovorum* subsp. *carotovorum* SCC1 and *P. atrosepticum* SCRI 1043 in dual cultures is shown on the left.

The *D. solani* bacteriocin operon is conserved with high nucleotide identity (83%) in *D. zeae* strains NCPPB 2538T and NCPPB 3531, whereas it is only partially conserved, albeit with high nucleotide identity, in *D. zeae* strain Ech586 and *D. dianthicola* strains IPO 980, NCPPB 453T and NCPPB 3534 that lack the first three genes in the operon (Figure 7). Interestingly, *D. zeae* strains NCPPB 2538T and NCPPB 3531 harbor a full-length operon similar to the *D. solani* bacteriocin cluster and also show growth inhibition activity against the SCC1 and SCRI1043 strains. In contrast, *D. zeae* Ech586 and all of the other *D. dianthicola* strains that lack the first part of the bacteriocin cluster are not able to inhibit the growth of *Pectobacterium* species (Figure 7). The association between the lack of the inhibition phenotype and the lack of the full-length bacteriocin cluster genotype suggested that the first part of the gene cluster might be essential for the production of the toxic molecule. Therefore, the *D. zeae* Ech586 and *D. dianthicola* strains were complemented with the first part of the bacteriocin cluster from *D. solani* D s0432-1; however, the inhibition activity was not restored in the complemented strains, which suggested that the missing DNA region might not be fully responsible for the lack of inhibiting ability. Other differences between orthologous bacteriocin gene clusters were investigated, including nucleotide insertions and deletions present in orthologs of ORF1003 in the *D. dianthicola* and *D. zeae* strains, respectively. However, complementation of

these nucleotide differences with the full-length gene from *D. solani* D s0432-1 did not lead to the acquisition of growth inhibition ability. Because the absence of the first three genes of the cluster and differences in the nucleotide sequences between *Dickeya* strains with different growth inhibition phenotypes were not sufficient to explain the lack of inhibiting activity, additional structural or regulatory genes outside the bacteriocin gene cluster may affect the production of the inhibiting molecule. An as yet unknown precursor peptide might be synthetized by a gene outside the bacteriocin operon, and the identified operon might be responsible only for posttranslational modification of the precursor peptide. Furthermore, if the leader and core peptides synthetized by different *Dickeya* species are dissimilar, the produced bacteriocins could be structurally different and consequently having different toxicity towards *Pectobacterium* cells.

The exact nature of the molecule produced by *D. solani* and presumably by some *D. zeae* strains remains unknown.

4.4.3. Bacterial competition on potatoes

The halo formation around *D. solani* colonies was visible in dual cultures with *Pectobacterium* strains only on MIM. This medium lacks rich nutrients and mimics plant apoplast nutrient conditions, suggesting that the same bacterial competition observed *in vitro* may also occur during infection of potato plants because many antibacterial compounds are produced when the bacteria experience nutrient shortages in order to maximize nutrient uptake at the expense of another competitor (Hibbing et al., 2010). Therefore, potato tubers were co-inoculated with *D. solani* wild type strain D s0432-1 and mutant strain M1001 in competition with *P. carotovorum* subsp. *carotovorum* SCC1, *P. atrosepticum* SCRI 1043 and *P. wasabiae* SCC 3193. Then, the bacteria in the rotten tissue was filtered and plated on CVP medium. The semi-selective CVP allowed the isolation of pectolytic bacteria that were tested for indole production from tryptophane. The indole-positive colonies were interpreted as *D. solani,* whereas the indole-negative colonies were interpreted as *Pectobacterium* species. The ratios between the *Dickeya* and *Pectobacterium* colonies isolated after potato tuber co-infections were analyzed using logististic regression test performed with SAS software, and the outcomes reflected the same bacterial interactions observed *in vitro*. *D. solani* D s0432-1 wild type was 5 and 23 times more likely to be isolated after tuber co-infections with *P. atrosepticum* and *P. carotovorum,* respectively, compared to the mutant strain M1001, confirming the greater ability of wild-type *D. solani* harboring the full-length bacteriocin cluster to overcome competitor strains. An increased number of *P. wasabiae* colonies was isolated after co-inoculation compared to the other *Pectobacterium* species, indicating that *P. wasabiae* was not as susceptible as the SCC1 and SCRI 1043 strains to the toxic compound produced by *D. solani*.

The ability to produce an antibacterial compound that is toxic to other closely related soft rot pathogens may positively affect *D. solani* competitiveness and establishment in the potato plant.

5. CONCLUDING REMARKS

In this study, the new species of plant pathogenic bacteria *D. solani* was characterized and the genes associated with its emergence were investigated. The new pathogen was first isolated from a diseased potato stem in Finland and identified as *Dickeya* biovar 3 strain using biochemical and PCR essays. Later, phylogenetic analysis confirmed that this strain belong to a highly clonal novel species named *D. solani* that has spread in Europe via the seed potato trade. A real-time PCR test was developed to speed up the identification of *Dickeya*-positive plants. This test allows the detection and quantification of all plant-infecting *Dickeya* species directly from plant tissue, excluding other closely related soft rot pathogens. *D. solani* appears to have exhibited an invasive character because it became in a short period of time the dominant soft rot potato pathogen in years with hot summers. Its high virulence leads to a reduced potato tuber yield due to rotting in the field and during storage. Yield loss and seed potato tuber downgrading due to *D. solani* infection represent a large economic problem that can be solved only by deepening the understanding of the ecology, pathogenesis and genomics of this new species to develop new diagnostics and other disease control strategies.

The study of the *D. solani* genome provided insights into major differences between this bacterium and the previously dominant *Dickeya* and *Pectobacterium* species that cause potato soft rot. *D. solani* harbors a unique combination of gene clusters that are involved in the production of secondary metabolites that may facilitate its establishment and predominance in the potato environment. Furthermore, the *D. solani* genome contains a gene cluster that is predicted to be involved in the production of a toxin similar to bacteriocin, thereby enabling this bacterium to inhibit the growth of previously dominant *Pectobacterium* species. The presence of this new gene cluster might explain the invasive character of *D. solani* and reinforce its ability to colonize and dominate the potato environment. However, further studies on the nature and toxicity of the produced bacteriocin are necessary to understand its mechanism of action and evaluate its employment in new disease management approaches.

ACKNOWLEDGEMENTS

This work has been carried out at the Department of Agricultural Sciences, University of Helsinki and funded by the Doctoral Program in Plant Science (University of Helsinki), the Agricultural and Forestry Fund (University of Helsinki) and the Department of Agricultural Sciences (University of Helsinki).

I wish to warmly thank my supervisor Dr. Minna Pirhonen for the constant guidance and edifying advices. I thank the members of my follow-up group Prof. Fred Asiegbu and Dr. Petri Auvinen for their support. I also want to thank Dr. Mirko Rossi and Professor Pablo Rodríguez Palenzuela for their precious comments and suggestions during the pre-examination of my thesis. All the co-authors from the publications included in my thesis are gratefully acknowledged.

A special thanks to all the colleagues from the Agricultural Sciences and Forest Sciences Departments for the fruitful discussions and for making this working experience very enjoyable.

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